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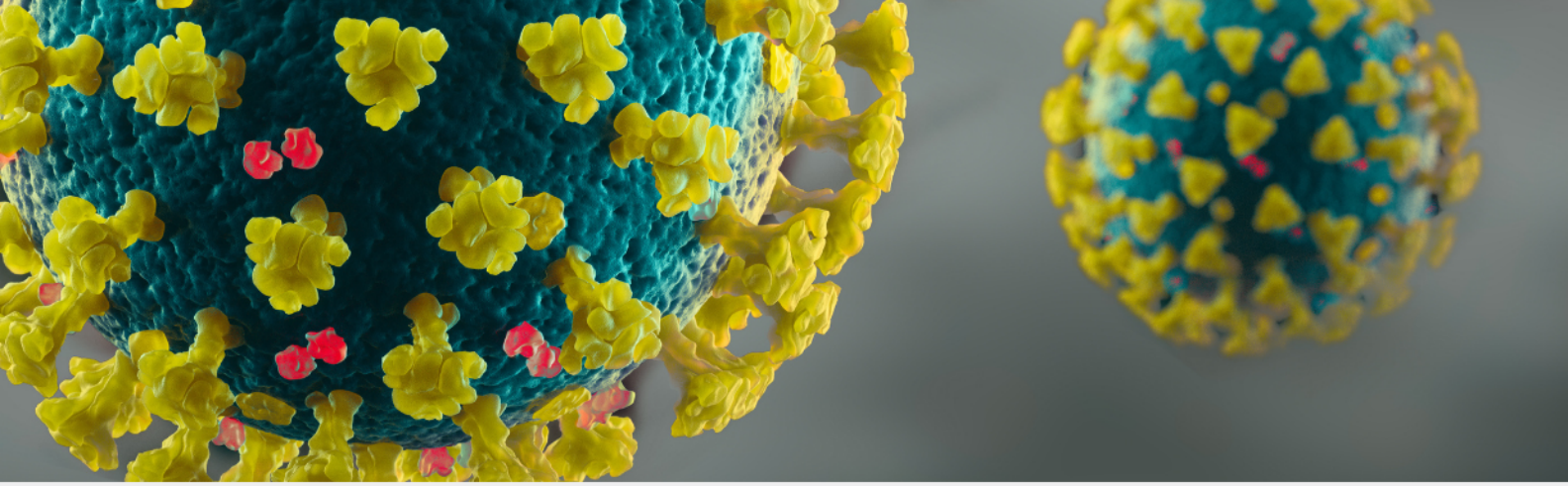

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Composition and stability of intestinal microbiota of healthy children within a Dutch population

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ABSTRACT **Numerous diseases linked to microbial imbalance can be traced back to childhood, illustrating the impact of the juvenile microbiota development from infancy toward adulthood. However, knowledge on this subject is currently very limited. The primary aim of this study was to characterize composition and short- and long-term stability of the intestinal microbiota in healthy children. Between November 2011 and June 2014, 61 children 2 to 18 yr of age from different areas in The Netherlands were included and instructed to collect fecal samples weekly, for 6 wk, and a follow-up sample after 18 mo. The intergenic spacer profiling technique (IS-pro) was used to analyze all available fecal samples. Microbial diversity was calculated by the Shannon diversity index and individual compositional stability by comparing all collection time points. Microbial stability varied per phylum ($P < 0.0005$), declined rapidly in a short time period, and subsequently stabilized on the long run with very gradual variation, leading to an overall compositional stability of 70% on average over a period of 18 mo. Higher species diversity was correlated to a higher overall compositional stability ($P < 0.001$). We observed an age-independent bacterial shared core consisting of a limited number of species. In conclusion, in this study, we showed that microbial composition stability in children varied per phylum, at both short-term and long-term intervals. Healthy children seem to share a microbiome core consisting of a limited number of species.—De Meij, T. G. J., Budding, A. E., de Groot, E. F. J., Jansen, F. M., Kneepkens, C. M. F., Benninga, M. A., Penders, J., van Bodegraven, A. A., Savelkoul, P. H. M. Composition and stability of intestinal microbiota of healthy children within a Dutch population. *FASEB J.* 30, 1512–1522 (2016). www.fasebj.org**

Key Words: core · 454-pyrosequencing · IS-pro · diversity · microbiome

The human body harbors trillions of microbes belonging to hundreds of different species, of which the vast majority reside in the gastrointestinal tract. Investigation into the pathophysiologic significance of our microbial symbionts has only recently been boosted by new DNA-based detection methods. It has become clear that the intestinal microbiota plays a crucial role in maintaining intestinal and overall health, including nutrient digestion and regulation of host metabolism and immune system (1–4). Disruption of the homeostasis between the gut microbiota and host has been linked to gastrointestinal and systemic diseases, such as inflammatory bowel disease, necrotizing enterocolitis, atopy, autoimmune diseases, and obesity (5–9). These observations have reinforced the potential of microbiota characterization as a diagnostic biomarker for health and disease, opening avenues toward development of novel, microbiota-targeted preventative and therapeutic strategies. For recognition of disease-specific microbial patterns and assessment of their role in (the course of) disease, detailed understanding of the intestinal microbiota in terms of composition and physiologic temporal variations is essential (10). Current knowledge on composition and dynamics of the human gut microbiota is limited, methodologically inconsistent, and largely based on studies in adults, describing a fairly stable composition over time. Studies on composition and temporal stability in children have almost exclusively focused on the first years of life, a period characterized by a highly dynamic and shifting intraindividual bacterial composition (11–13). Information on the development and stability of the microbiota from infancy toward adulthood is yet very limited. The fact that numerous

Abbreviations: FAFV, Firmicutes, Actinobacteria, Fusobacteria, and Verrucomicrobia; IS, intergenic spacer; OTU, operational taxonomic unit

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diseases linked to microbial imbalance can be traced back to this specific period illustrates the importance and impact of the juvenile microbiota (14–16). Characterization of the normal microbiota in children and its development from infancy toward adulthood is central to enlarge knowledge about microbiota-related pathology.

The primary aim of this study was to characterize composition and short-term and long-term stability of the intestinal microbiota in healthy children from 2 to 18 yr of age.

MATERIALS AND METHODS

Subjects and study design

A heterogeneous cohort of healthy Dutch children was included to represent a cross section of children from all ages. For this purpose, we approached parents of children in the age range of 2–18 yr, visiting primary and secondary schools in both urban and rural areas in 5 different provinces of the Netherlands (Noord-Holland, Zuid-Holland, Overijssel, Friesland, and Flevoland), and invited them to participate. To focus exclusively on temporal gut microbiota dynamics during state of health, strict criteria for exclusion were applied: use of antibiotics or immune modulating agents within 6 mo prior to the study, culture-proven infectious gastroenteritis in the last 6 mo prior to inclusion, history of surgery of the gastrointestinal tract (except appendectomy), or a diagnosis of chronic gastrointestinal disease, such as celiac disease, functional constipation, short bowel syndrome, or inflammatory bowel disease. Subjects (or their parents) completed a questionnaire on the following items: age, length and weight, pregnancy duration, mode of delivery, type of neonatal feeding (breastfeeding or formula feeding), use of antibiotics in the first year of life, current medication, and defecation pattern.

This study was approved by the Medical Ethics Committee of VU University Medical Center and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Sample size calculation

A formal power analysis could not be performed for this study because no previous data on microbiota stability in healthy children were available.

Sampling

Sterile plastic containers and an information letter were provided to parents and children, with instructions on procedure of collection and storage of the fecal samples. Children (or their parents) were instructed to collect fecal samples (approximately 2 g) for 6 wk at weekly sampling intervals and a follow-up sample after 18 mo. Fresh fecal samples were immediately stored in the freezer at home at −20°C. For collection of the 18-mo follow-up samples, parents were contacted by telephone or e-mail. Nonresponders received a maximum of 2 reminders. At time of collection of the follow-up fecal sample, information was obtained on use of antibiotics and major changes in health status during the last 18 mo, such as a diagnosis of (chronic) gastrointestinal disease. DNA extraction was performed after collection of those frozen fecal samples collected at weekly intervals, and DNA extraction of the follow-up samples was performed within 3 mo following collection.

DNA extraction and sample preparation

DNA was extracted from fecal samples with the easyMag extraction kit according to the manufacturer's instructions (Biomérieux, Marcy l'Etoile, France). Feces (100–400 mg) was placed in an Eppendorf tube with 200 μl of nucliSens lysis buffer and vortexed. Tubes were incubated while shaking for 5 min at room temperature. After centrifugation (13,000 rpm; 2 min), 100 μl supernatant was transferred to an easyMAG isolation container containing 2 ml nucliSENS lysis buffer. This suspension was incubated for 10 min at room temperature, after which 70 μl of magnetic silica beads were added. The easyMAG automated DNA isolation machine was used following the "specific A" protocol, eluting DNA in 110 μl buffer. All fecal samples were analyzed by intergenic spacer profiling (IS-pro), and the first samples collected (baseline samples) were also analyzed by 454-pyrosequencing (17).

IS-pro

For IS-pro, DNA samples were diluted 1:10. Amplification of IS regions was performed with the IS-pro assay (IS-Diagnostics, Amsterdam, The Netherlands) according to the protocol provided by the manufacturer. IS-pro differentiates bacterial species by the length of the 16S–23S rDNA IS region with taxonomic classification by phylum-specific fluorescently labeled PCR primers (17). The procedure consists of 2 PCR reactions, together covering the phyla Firmicutes, Actinobacteria, Fusobacteria, Verrucomicrobia, Bacteroidetes, and Proteobacteria. The first PCR reaction contains 2 different fluorescently labeled forward primers and 3 unlabeled reverse primers. The first forward primer is specific for the phyla Firmicutes, Actinobacteria, Fusobacteria, and Verrucomicrobia (FAFV), and the other labeled forward primer is specific for the phylum Bacteroidetes. The second PCR contains a forward-labeled primer specific for the phylum Proteobacteria and a combination of 7 reverse primers, together covering the phylum Proteobacteria.

The IS-pro primers have been extensively evaluated for coverage of the various phyla included in the assay. An *in silico* analysis was performed on all 16S–23S sequences available at GenBank in April 2014 (8990 sequences). Here, matches for both forward and reverse primers were found in 95% of Firmicutes (3129/3303), 99% of Actinobacteria (814/815), 88% Bacteroidetes (245/280), and 98% of Proteobacteria sequences (3714/3803). Furthermore, to confirm *in silico* results and to evaluate reproducibility of IS profiles within species, IS-pro reactions were performed on 588 cultured bacterial strains, belonging to 189 species from the phyla covered by the IS-pro primers. These findings confirmed *in silico* predictions. The data from the *in silico* and *in vitro* analyses were combined to form a database used in translation of profiles to species names.

Amplifications were carried out on a GeneAmp PCR system9700 (Applied Biosystems, Foster City, CA, USA). After PCR, 5 μl PCR product was mixed with 20 μl formamide and 0.2 μl custom size marker (IS-Diagnostics). DNA fragment analysis was performed on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems).

454-Pyrosequencing

Amplicon libraries for pyrosequencing of the 16S rDNA V1–V3 regions were generated using 2 primers: the first primer was a barcoded reverse primer consisting of the 454 Titanium platform A linker sequence (5′-CCATCCCTGCGTGTCTCCGACTCAG-3′), a key (barcode of 6–8 nt as described by the HMP consortium in *http://www.hmpdacc.org/doc/16S_Sequencing_SOP_4.2.2.pdf*) that was unique for each sample, and the 16S rRNA 534R primer sequence 5′-ATTACCGCGGCTGCTGG-3′. The second primer was a forward primer consisting of a 9:1 mixture of 2 oligonucleotides, 5′-B-AGAGTTTGATCMTGGCTCAG-3′ and 5′-B-AGGGTTCGATTCTGGCTCAG-3′, where B represents the B linker (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′) followed by the 16S rRNA 8F and 8F-Bif primers, respectively (18).

PCR amplifications (in a volume of 50 μL) were performed using 1× FastStart High Fidelity Reaction Buffer, 1.8 mM $MgCl_2$, 1 mM dNTP solution, 5 U FastStart High Fidelity Blend Polymerase (from the High Fidelity PCR System; Roche, Indianapolis, IN, USA), 0.2 μM reverse primer, 0.2 μM of the barcoded forward primer (unique for each sample), and 1 μL of template DNA. PCR cycle conditions were as follows: an initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s, and extension at 72°C for 5 min, and a final elongation step at 72°C for 10 min. Amplicons (20 μL) were purified using AMPure XP purification (Beckman Coulter, Beverly, MA, USA) according to the manufacturer's instructions and eluted in 25 μl TE buffer 1× solution, pH 8.0, and low EDTA.

Amplicon concentrations were determined by the Quant-iT PicoGreen dsDNA reagent kit (Thermo Fisher Scientific, Grand Island NY, USA) using a Victor3 Multilabel Counter (Perkin Elmer, Waltham, MA, USA). Amplicons were mixed in equimolar concentrations. 454-Sequencing was performed on a GS FLX Titanium PicoTiterPlate with a GS FLX pyrosequencing system (Roche, Branford, CT, USA).

Data analysis

IS-Pro

Preprocessing was carried out with the IS-Pro proprietary software suite (IS-Diagnostics) and resulted in microbial profiles. Three levels of information were obtained: color of peaks sorts species into the phyla FAFV, Bacteroidetes, and Proteobacteria, which are the main phyla present in the human gastrointestinal tract. Length of the 16S–23S rDNA IS region, displayed by number of nucleotides, can subsequently be used to identify bacteria at the species level. Specific peak height, measured in relative fluorescence units, reflect the quantity of PCR product. To further analyze the obtained data, each peak in a profile was considered as an operational taxonomic unit (OTU) and its corresponding intensity as its abundance. Species determination of IS-pro peaks was done by matching of profiles to a database of IS profiles of known bacterial species. In previous analyses, the effect of sampling on IS profiles was assessed. The correlation of profiles from samples of the same excrement was found to be 96% for Bacteroidetes and 90% for FAFV (17).

454-Pyrosequencing

Raw pyrosequencing reads were passed through quality filters to reduce the overall error rate using Mothur version 1.23 (19). Only sequences with perfect proximal primer fidelity and a threshold quality score of ≥20, a read length between 200 and 540 nucleotides, a maximum of 1 ambiguous base call, and a maximum homopolymer length of 6 were retained for further analysis. Data processing was done with Quantitative Insights Into Microbial Ecology, version 1.5.1 (20). Barcodes were used to identify sequences from each individual sample. The UCLUST algorithm was used to cluster sequences into OTUs or phylotypes based on 97% similarity (species level) against the Greengenes reference set (21). The following nondefault search parameters for Uclust algorithm were applied: maxrejects = 100 and stepwords = 16. Creation of new clusters for sequences that did not cluster to reference sequences within the given similarity threshold was disabled to further reduce the influence of pyrosequencing errors. Finally, OTUs that were only recovered in a single sample were removed from the dataset. In total, 1,403,456 sequences were recovered from 454 sequencing. After trimming, filtering, binning, and clustering, 722,109 sequences (mean ± SD length: 326 ± 76.1 bases), ranging from 6195 to 18,662 sequences per sample, remained for further analysis. For downstream analyses, data were rarefied at a sequencing depth of 6195 sequences per sample.

Diversity and stability analysis

Diversity and stability analysis was performed on the IS-pro data. Microbial diversity was calculated as the Shannon diversity index based on the resulting profiles by conventional statistics. Diversity was calculated both per phylum and for overall microbial composition (by pooling the phyla FAFV, Bacteroidetes, and Proteobacteria together). Diversity analysis was performed with the R 2.15.2 software package. Data visualizations and fitted curves were done with the Spotfire software package (Tibco, Palo Alto, CA, USA).

In this study, compositional stability is defined as intra-individual resistance to change in relative abundances of species over time, quantified by cosine distance (lower distance value represents higher stability), and expressed as a percentage value (*e.g.,* when 2 fecal samples of 1 individual collected over time would have identical microbial composition, compositional stability is 100%). The compositional stability of fecal microbiota of children through time was estimated by comparing all intervals per individual (*i.e.,* for 1 week stability, all 1-wk intervals were compared; for 2-wk stability, all 2-wk intervals were compared and so on). For 18 mo stability, the follow-up sample was compared with all other samples to minimize impact of potential outliers on this analysis. Sample compositions were compared by calculating cosine distances for log2-transformed data per phylum and for the phyla FAFV, Bacteroidetes, and Proteobacteria combined (17). Multivariate ANOVA was performed, when appropriate using a Greenhouse-Geisser correction, taking time and phylum as within-subject variables. Dissimilarities between samples, or between-sample diversity, were represented in a dissimilarity matrix that was built using the cosine distance measure. Given 2 vectors of attributes (2 profiles in our case), A and B, the cosine dissimilarity is represented using a dot product and magnitude as

$$\text{Dissimilarity} = 1 - \cos(\theta) = 1 - \frac{\sum_{i=1}^{n} A_i \times B_i}{\sqrt{\sum_{i=1}^{n} (A_i)^2} \times \sqrt{\sum_{i=1}^{n} (B_i)^2}}$$

Network analysis

To analyze the potential presence of a microbial core at the level of OTU, a concept defined as a fixed set of bacterial species present in more than 80% of all healthy children, a network visualization approach was taken. An anchored bipartite network was generated from all IS-pro data (22). Samples were represented as anchored nodes in a circular layout and bacterial OTUs as unanchored nodes for which the position was calculated as the center of mass of all connected sample nodes. Edges were created between a sample and an OTU node when that particular OTU was present in that particular sample. OTUs that were present in only a single sample were placed outside the circle of anchored sample nodes. The network visualization was made in Cytoscape 3.1.0 (23). For this particular visualization, a custom plugin was made. The color of nodes was determined by degree and the size by betweenness centrality (24). Finally, edges were bundled with the "bundle edges" plugin. Parameters were as follows: number of handles, 3; Spring constant, 0.003; compatibility threshold, 0.3; maximum iterations. For an illustrated step-by-step description of how the network was built, refer to Supplemental Data.

RESULTS

Participants

Between November 2011 and June 2014, 61 children were included in this study; each child collected 1 fecal sample

per wk for 6 wk. Forty-five children also collected a follow-up sample after 18 mo. None of the children and parents reported relevant changes in health state between collection of first samples and follow-up sample; 2 children used antibiotics for a respiratory tract infection. All children reported normal defecation patterns with more than 3 bowel movements per wk. All parents were born in Western Europe, except for 3 couples with diverse backgrounds (South Korea, Aruba, and Peru). The characteristics of the participants are summarized in **Table 1**.

Diversity and stability

Microbial compositional stability through time varied per time point ($P < 0.0005$) and per phylum ($P < 0.0005$), with Bacteroidetes showing the highest compositional stability, followed by Proteobacteria and FAFV. Stability declined quite rapidly for short intervals, but then stabilized at a level that declined further only very gradually. P values for longitudinal decrease were 3.41×10^{-27} for Proteobacteria, 5.32×10^{-19} for Firmicutes, and 1.33×10^{-14} for Bacteroidetes. Overall compositional stability was on average 70% over a period of 18 mo (**Fig. 1**). Also at the level of the individual, it was found that Bacteroidetes composition was most stable, whereas the compositional stability of FAFV and Proteobacteria was lower on average. Finally, there seemed to be a slight increase in compositional stability with age, but this effect was not statistically significant (**Fig. 2*A***). The identified variations in microbiota composition through time far exceeded the expected variation induced by multiple testing (*i.e.,* taking a sample from the same source twice and performing the whole analysis procedure twice, from DNA isolation to final data analysis), indicating that the measured effect was indeed temporal variation.

TABLE 1. *Subject characteristics*

Characteristic	Value
Number of subjects (n)	61
Age [median (IQR)] (yr)	7.8 (6.7)
Range (yr)	2.1-17.8
Male (%)	48
18-mo follow-up sample (n)	45
BMI [median (IQR)]	15.9 (3.6)
Area inclusion[a] (n)	
Agriculture	8
Urban	53
Mode of delivery (n)	
Vaginal	53
Caesarian delivery	8
Pregnancy duration (n)	
<37 wk	3
37–42 wk	58
Neonatal feeding (n)	
Exclusively formula fed	1
Breastfeeding	60
Duration of breastfeeding (n)	
<3 mo	10
3–6 mo	26
>6 mo	24
Antibiotic use first year of life (n)	13
Medication at time of sampling (n)	
Probiotics	6
Birth control pills	1
Salbutamol inhalator	2

BMI, body mass index; IQR, interquartile range. [a]Urban area includes towns and cities; children from agriculture origin live on a farm.

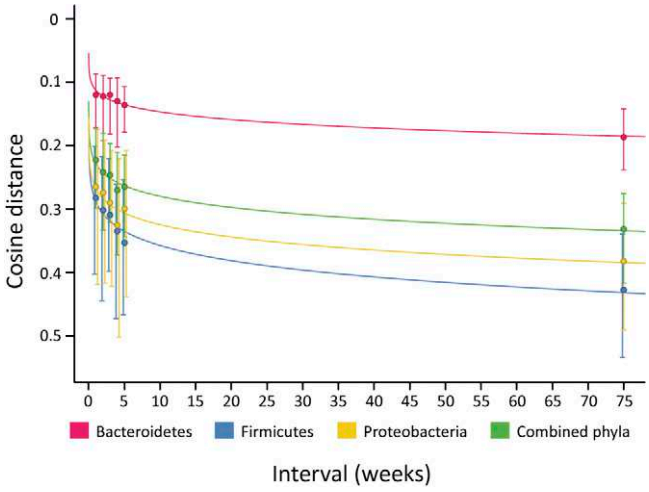

Figure 1. Compositional stability of fecal microbiota of children through time. Dots represent median cosine distance of microbiota composition (y axis) per time interval (x axis). Microbiota stability declined quite rapidly for short intervals, but then stabilized at a level that declined further only very gradually, with the highest stability for phylum Bacteroidetes (red), followed by Protebacteria (yellow), and FAFV (blue). Overall compositional stability is depicted in green.

Diversity of fecal samples was highest for the phylum Bacteroidetes with a median Shannon diversity index of 2.81, followed by the phyla Proteobacteria (2.62) and FAFV (2.46) at baseline. Stability of diversity indices over time was also assessed and was found to be highest for the phylum Bacteroidetes (**Fig. 3**). Diversity index for the phyla FAFV and Proteobacteria appeared to be more variable over time. A further assessment was done to analyze microbial diversity, its stability, and relation to age on an individual level. The results are shown in Fig. 2*B*. Here, diversity was highest and most stable over time for the phylum Bacteroidetes. For the phyla FAFV and Proteobacteria, median diversity indices and individual variation were much larger than for the phylum Bacteroidetes.

Importantly, compositional stability and stability of diversity of the measured phyla FAFV, Bacteroidetes, and Proteobacteria were highly individual specific. A higher diversity was correlated with higher stability for these phyla separately and for the combined microbiota. Also, higher diversity was correlated to a higher stability of the diversity index (**Fig. 4**). Children exposed to antibiotics in their first year of life had similar Shannon diversity indices compared with unexposed children (data not shown).

When allocating children to 1 of 3 defined age groups (blocks of 5 yr: from 2 to 7 yr of age; from 8 to 13 yr; and from 13 to 18 yr), no statistically significant differences were observed in composition, stability, and diversity between these 3 age cohorts with an unpaired Student's t test. Furthermore, no particular clustering was seen based on sex.

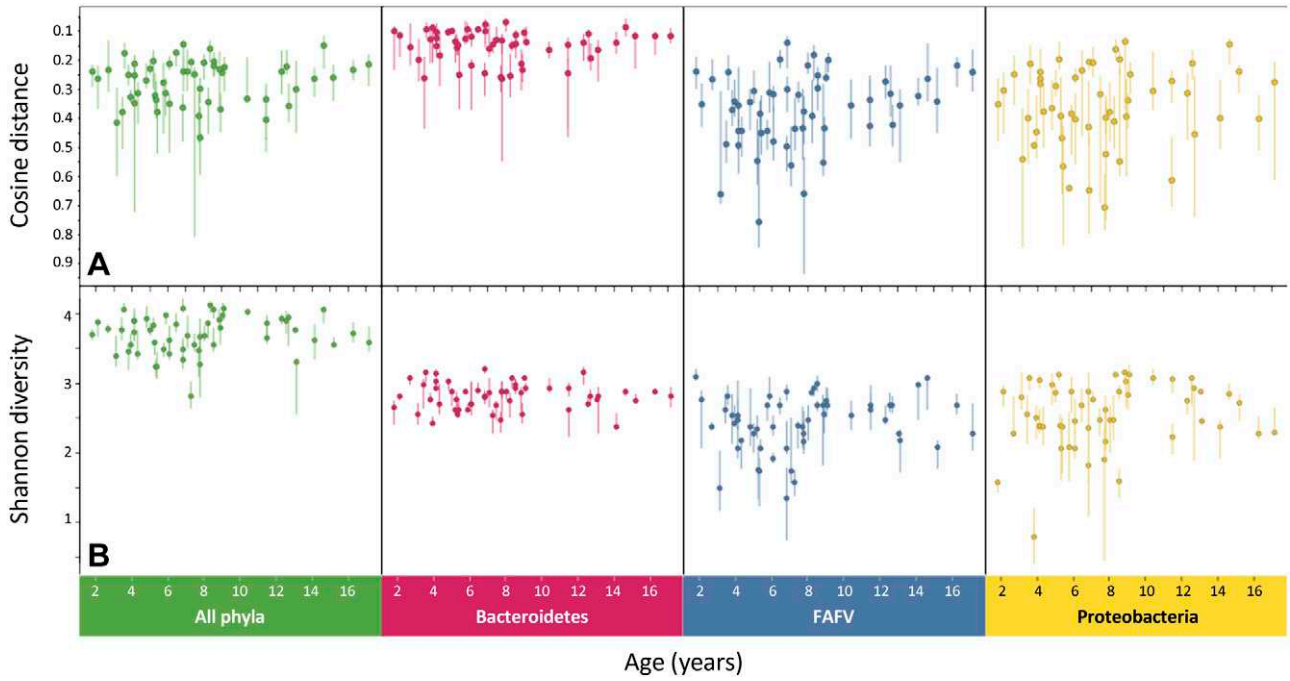

Figure 2. Individualized analyses on IS-pro data for the phyla Bacteroidetes (red), FAFV (blue), Proteobacteria (yellow), and all phyla (green), sorted by age (in years, *x* axis). Dots represent median values of all samples from a given child, except the 18 mo follow-up sample, plotted as function of age in years. Error bars represent interquartile range (IQR). *A*) Compositional stability per child sorted by age as expressed by cosine distance between sequential samples (lower values represent higher stability). Compositional stability was highest for Bacteroidetes, whereas the stability for the other phyla appeared more individualized: especially in younger children, interindividual variation in stability of FAFV and Proteobacteria was high. *B*) Diversity was highest and most stable for the phylum Bacteroidetes. For the phyla FAFV and Proteobacteria, diversity indices had a larger distribution and were less stable.

Microbiota core

In the network analysis, a bacterial core consisting of species that were present in the fecal samples of the majority of children became apparent (**Fig. 5**). This core was dominated by species from the phylum Bacteroidetes, with the genera *Bacteroides* and *Alistipes* having the highest average abundances. From the phylum Firmicutes, the families Ruminococcacaea and Lachnospiraceae were most dominant. The presence and identity of this shared core was confirmed in all baseline samples with 454-pyrosequencing (**Fig. 6**). **Table 2** summarizes the most abundant OTUs in 454 data (present in >70% of all subjects) and their matching to the most common species as found with IS-pro. **Figure 7** represents IS-pro and 454-sequencing data of the most abundant taxa at the family level, including their relative abundance.

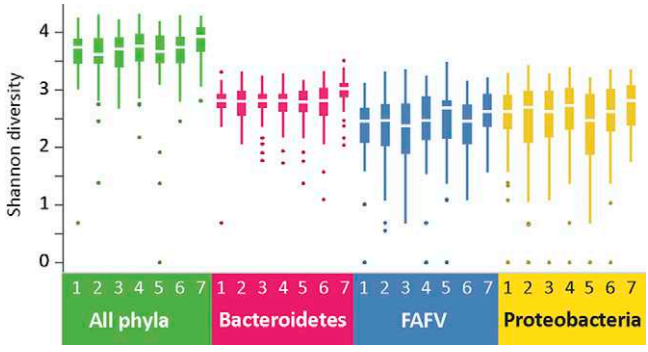

Figure 3. Boxplot of Shannon diversity indices at different time points (T1–6 correspond with sampling number in weeks, T7 is follow-up at 18 months). Bacteroidetes had the highest and most stable diversity at all time points, followed by Proteobacteria and FAFV. Distribution of diversity was considerably larger for the latter phyla than for Bacteroidetes. Notably, at T7, Bacteroidetes diversity seemed to be increased for all children.

DISCUSSION

In this study, we showed that microbial composition stability in children is individualized and varied per phylum at both short-term and long-term intervals. This was consistent throughout all age cohorts of youth. Diversity and stability for all bacterial phyla were correlated: diversity of the phylum Bacteroidetes was highest, and this phylum also showed the highest stability compared with the phyla Proteobacteria and FAFV. Finally, we observed the presence of a shared microbial core, consisting of a limited number of species that was present in the majority of healthy children.

Dynamics

Human gut microbiota stability patterns were first described in 1998 by Zoetendal *et al.* (25). They showed that the composition of the gut microbiota of 2 adults remained fairly stable over a course of months. Subsequent studies

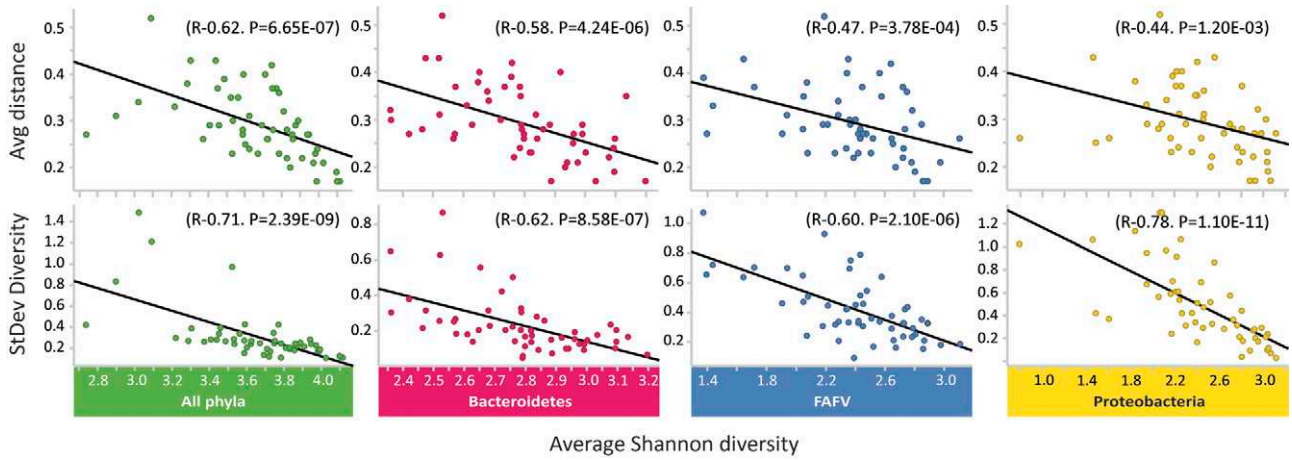

Figure 4. Correlation of average diversity values per child with average cosine distances (*upper*) and standard deviation of Shannon diversity index (*lower*) for the phyla FAFV, Bacteroidetes, and Proteobacteria together (green) and Bacteroidetes (red), FAFV (blue), and Proteobacteria (yellow) seperately. A significant negative correlation was apparent in both analyses for all measured phyla. Children with a higher diversity of their microbiota thus showed a lower variation of that diversity through time, as measured by the standard deviation of diversity, Also, samples with higher diversity are compositionally more similar throughout time, as measured by the average cosine distance between all time points. Spearman rank correlation coefficients and associated *P* values are depicted for each correlation.

confirmed the existence of relatively stable, host-dependent microbiota, characterized by prominent fluctuations around the average, at both species and phylum levels (26–34). However, this generally stable course has been shown to be affected by lifestyle and major events in human life, such as dietary alterations, enteric infections, and traveling from the developed to the developing world, which could rapidly and profoundly impact microbiota dynamics (35). More recently, 2 studies including 5 and 37 healthy adults, respectively, showed that stability of the gut microbiota differed per phylum (36, 37). In adults, the composition of Bacteroidetes and Actinobacteria was more stable and that of Proteobacteria was less stable over time compared with overall stability. Firmicutes showed a more variable pattern, consisting of both highly stable and less stable members. In our study including 61 children, we also observed that stability varied per phylum, with the phylum Bacteroidetes as the most stable component of the gut microbiota, followed by the Proteobacteria and FAFV. Similar to the observations in adults by others, we noticed that microbiota stability in children between 2 and 18 yr of age declined quite rapidly for short intervals, but then stabilized at a level that declined further only very gradually. In adults, on average, 70% and 60% of identified strains were preserved over the course of 1 and 5 yr, respectively. We found an overall compositional stability of on average 70% over a period of 18 mo.

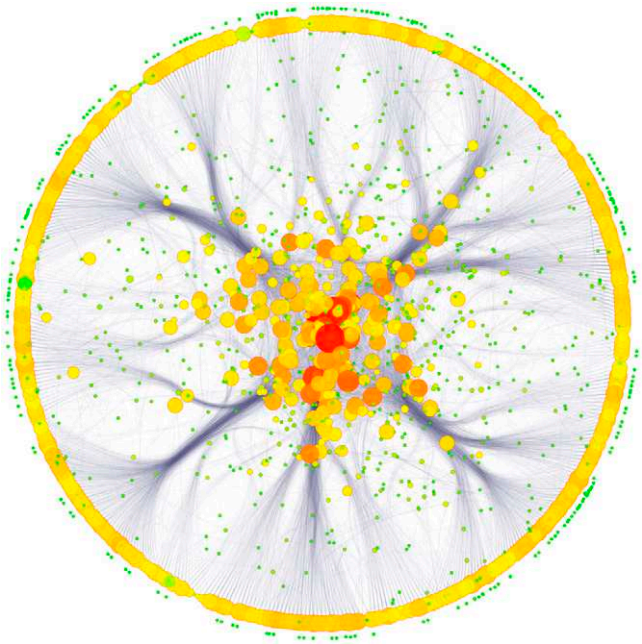

Figure 5. Anchored bipartite network depicting samples from all children at all time points in a circular layout, based on all IS-pro data. All samples of all children are displayed as nodes on the circumference of the circle. Bacterial OTU nodes were placed at the center of gravity of the sample nodes in which the OTUs occur. Thus, the more children who shared a certain OTU, the more central that OTU has been placed. Bacteria that were common in many individuals appeared as a visual core. Size and color of nodes were determined by the number of nodes these were connected to (red, highest connectivity; green, lowest connectivity). Core species appeared in the center, were larger, and had a hue toward red.

Phylum-specific temporal shifts in composition may be due to variation in individual diets, because most prominent fluctuations have been described in bacterial species involved in food digestion, such as *Bifidobacterium adolescentis* and *Parabacteroides distasonis*, and possessing carbohydrate-degrading enzymes, such as *Clostridium clostridioforme* and *Faecalibacterium prausnitzii* (38). This concept of dietary effects on intraindividual microbial dynamics was supported by a study on temporal compositional stability that compared Bangladeshi and U.S. children (39).

Remarkably, we noticed an increase in diversity index of the phylum Bacteroidetes at 18 mo of follow-up, whereas no increase was observed for the other phyla. In this

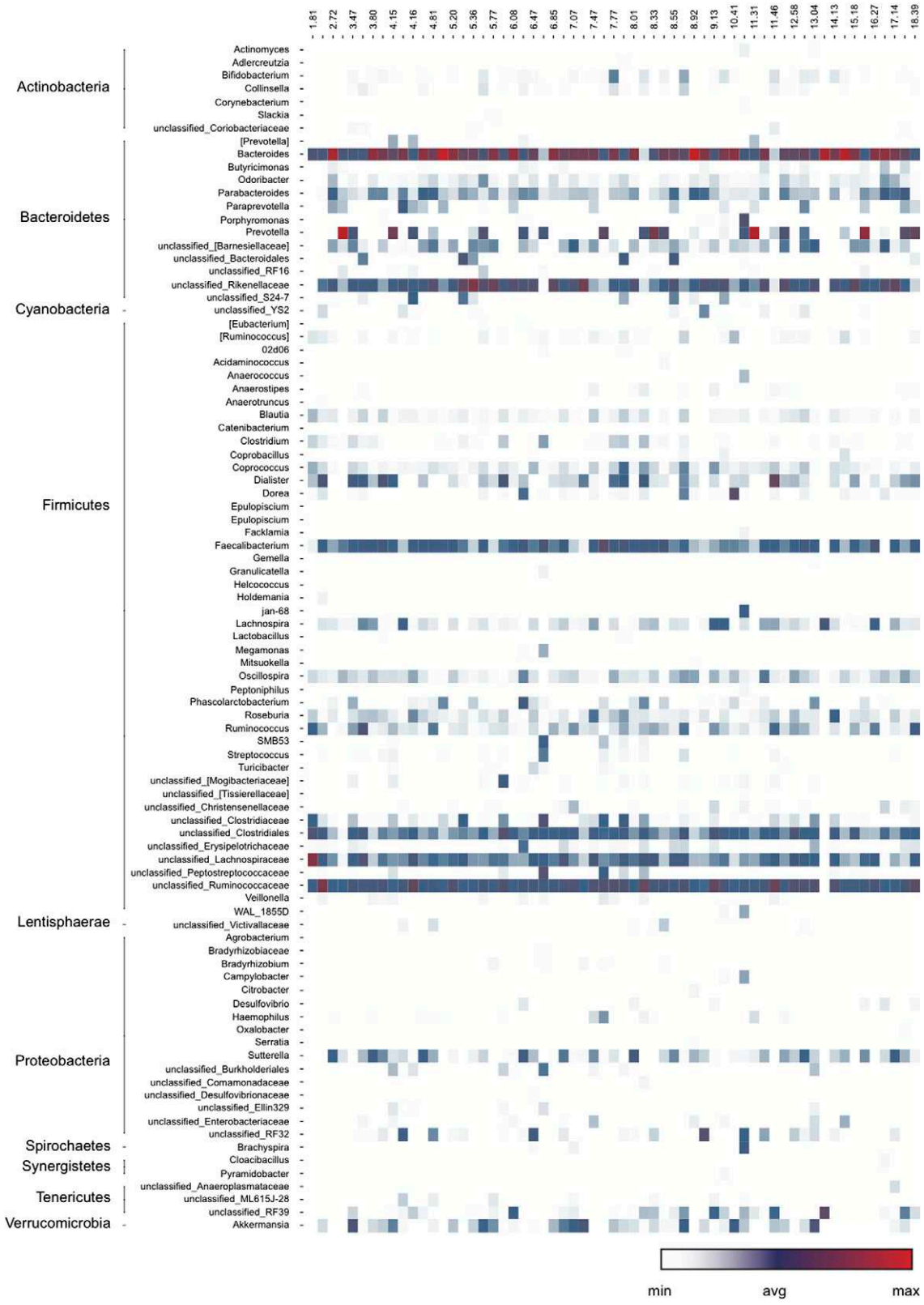

Figure 6. Heatmap of 454-pyrosequencing data of all baseline samples, sorted by age (on *x* axis), with different species on *y* axis. Only taxa with an abundance >0.1% are displayed. Intensity of colors reflects relative abundance in each sample. It can be clearly seen here that some genera occur in almost all individuals (shown as horizontal lines). These genera correspond with the core species as detected by IS-pro data.

TABLE 2. *Matching of 454-sequencing and IS-pro data for identification of core species*

Core species	454-Sequencing [prevalence (%)]	IS-pro peaks
Alistipes finegoldii	60/61 (98)	231/396/400/406
Faecalibacterium prausnitzii	60/61 (98)	313/318/320
Bacteroides vulgatus	59/61 (97)	479
Alistipes putredinis	58/61 (95)	235
Bacteroides fragilis	58/61 (95)	537
Prevotella spp.	58/61 (95)	437
Odoribacter splanchnicus	56/61 (92)	308
Parabacteroides distasonis	54/61 (89)	464
Sutterella wadsworthensis	53/61 (87)	660–663/871
Unclassified Ruminococcaceae	53/61 (87)	888
Unclassified Firmicute	51/61 (84)	257
Sutterella spp.	50/61 (82)	651
Unclassified Proteobacteria	48/61 (79)	941
Unclassified Firmicute	48/61 (79)	361
Escherichia coli	47/61 (77)	735/828
Bacteroides spp.	46/61 (75)	474
Bacteroides spp.	45/61 (74)	548
Unclassified Firmicute	45/61 (74)	541
Akkermansia muciniphila	43/61 (70)	598/602
Unclassified Firmicute	43/61 (70)	558
Unclassified Proteobacteria	43/61 (70)	747

The most abundant OTUs as detected by 454-pyrosequencing data and their matching to the most common species as found with IS-pro are displayed, including a column displaying prevalence of each taxon.

respect, it should be noted that the majority of 18 mo follow-up samples were collected during the summer, whereas the first weekly samples were collected mainly during the winter. Seasonal shifts in microbiota composition, including shifts in Bacteroidetes, have recently been described in adults, and have been attributed to seasonal dietary differences (40).

We also observed a positive correlation between diversity and stability. This finding is of general ecological interest as this has been a long debated issue for nonmicrobial ecosystems (41). Furthermore, this finding may also be of particular interest for the role of microbiota in health and disease, because low microbial diversity has been linked to various diseased states (42). It is interesting to observe that stability of diversity may be higher than compositional stability, as we found for the phylum Proteobacteria. This suggests succession of different species within the population while maintaining the same level of diversity.

Composition and core

Until recently, it has been assumed that at the age of about 3 yr, the gut microbiota composition has converged toward a relatively stable, adult-like pattern (11, 43, 44). In 2 recent studies, however, substantial compositional differences between children of different ages and adults were described, suggesting that, also from a microbial point of view, children cannot be regarded as miniaturized adults (45, 46). In the present study, we observed a slight, but not statistically significant, increase in compositional stability with age.

The presence of a dominant microbial core, usually defined as a set of phylotypes shared by the majority of subjects, has been considered in several studies (38, 47). The search for such a core has been a major target of the human microbiome project (48). Data on the existence of a shared, temporal core are contradictory, due to differences in definition of what is considered a common core and differences in depth of the analysis. Depth of the analysis is particularly important when studying phylogenetic groups with low abundances (49, 50). Two recent studies based on whole metagenome sequencing provide further evidence for the presence of a shared microbiome core in adults, with Actinobacteria, Bacteroidetes, and Verrucomicrobia populations as the most stable members (38, 51). In contrast, it has been reported that only a small fraction of all taxa found within a single body was constantly present over time, suggesting that a core temporal microbiome of high abundant taxa does not exist (52). In concordance, in a study describing intestinal microbiota composition in 154 adults, no single bacterial phylotype was present at an abundant frequency in fecal samples of all included subjects. Instead, the authors concluded that a core gut microbiome seems to exist at the level of metabolic functions (48). In the present study, we observed the presence of a shared microbial core in children, independent of age, dominated by species from the phylum Bacteroidetes, with the genera *Bacteroides* and *Alistipes* as the most abundant members.

The strengths of this study were the large size of the cohort, the number of fecal samples per individual, and that microbial analysis was performed using 2 different bacterial DNA-specific techniques, IS-pro and 454-pyrosequencing, allowing us to cross-validate our findings. Observed species present in the shared core as measured by IS-pro were similar to core microbes established by 454-pyrosequencing. Although our cohort has characteristics of heterogeneity, because healthy children aged 2–18 yr

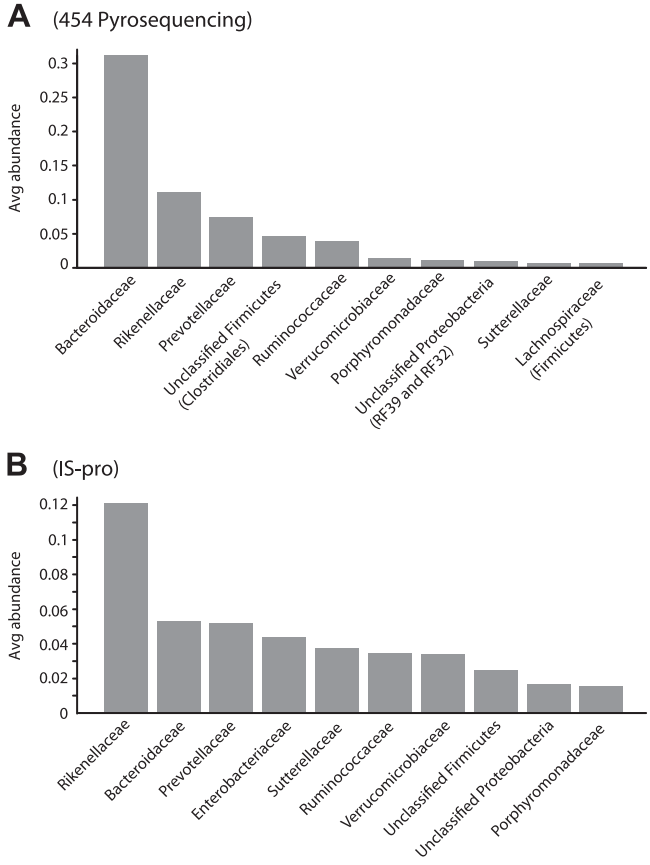

Figure 7. Bar chart representing data by 454-sequencing (*A*) and IS-pro (*B*) of most abundant taxa at the family level. Although observed abundances are not identical, the detected families are largely the same. A notable difference is the relatively high abundance of the family Enterobacteriaceae (mainly *E. coli*) in the IS-pro data.

are heterogeneous, we examined subgroups based on age and sex. Because findings in stability and composition did not differ between sex or the 3 age cohorts, we assumed the presented data to be generalizable to healthy (Dutch) children overall: the limited standard deviation in the collected specimens indicated that the number of analyzed samples was sufficient to draw universal conclusions. The present study also had limitations; the influence of environmental factors on microbiota composition, in particular later in childhood, such as antibiotic use during the first year of life, way of delivery, and neonatal feeding pattern, could not be assessed. Specifically, formula-fed children were underrepresented in this study. Additionally, food intake was not recorded daily during the study period, because detailed understanding of the influence of day-to-day changes in diet on temporal microbial dynamics would need a cohort consisting of at least hundreds of subjects; thus, the current series of children was too small for suitable statistical analysis addressing influence of diet (53, 54).

Future perspectives

We showed that gut microbiota composition in children is not rigid but subject to prominent fluctuations even at intervals of weeks. Presented compositional variability in health may further increase understanding of the role of the intestinal microbiota in the course of pediatric diseases linked to microbiota alterations, such as inflammatory bowel disease.

Furthermore, the observed intraindividual dynamics may have implications for future strategies assessing healthy individuals' gut microbiota signature. The currently used single-sampling strategy to analyze microbiota composition in population-wide (pediatric) studies, but also in single individuals, might not accurately reflect the entire compositional variability over time. Determination of the average composition measured at different time points could offer a more integral view. Such an approach of multiple sampling is common practice in the diagnostic workup of parasitic infections, in which a triple feces test is performed to enhance the sensitivity and specificity of the test. Regarding the observed shared microbiome core, future studies are needed to establish its precise role in health and whether disruption is linked to (increased risk for) disease and to assess environmental factors potentially influencing core composition.

In summary, in the largest study thus far on the short-term and long-term stability of the intestinal microbiota in healthy children between 2 and 18 yr of age, we showed that stability in children is an individual characteristic varying per phylum at both short-term and long-term intervals. Stability declined rapidly for short intervals but stabilized at a level that declined further only very gradually. This was consistent throughout all age cohorts of youth. FJ

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